

Aestuariimicrobium kwangyangense gen. nov., sp. nov., an LL-diaminopimelic acid-containing bacterium isolated from tidal flat sediment

Seo-Youn Jung, Hee-Sik Kim, Jae Jun Song, Seung-Goo Lee, Tae-Kwang Oh and Jung-Hoon Yoon

Korea Research Institute of Bioscience and Biotechnology (KRIBB), PO Box 115, Yusong, Taejeon, South Korea

Correspondence

Jung-Hoon Yoon
jhyoona@kribb.re.kr

Four Gram-positive, catalase-positive, short rod- or coccoid-shaped bacterial strains, R27^T, R44, R45 and R47, were isolated from an enrichment culture with diesel oil-degradation activity and their taxonomic positions were investigated using a polyphasic approach. Phenotypic, phylogenetic and genetic similarities indicated that strains R27^T, R44, R45 and R47 belong to the same species. Phylogenetic analysis based on 16S rRNA gene sequences showed that the four strains form a distinct evolutionary lineage within the family *Propionibacteriaceae*. The novel four strains had cell-wall peptidoglycan based on LL-diaminopimelic acid, MK-9(H₄) as the predominant menaquinone and anteiso-C_{15:0} as the major cellular fatty acid. The DNA G + C contents were 68.8–69.2 mol%. These chemotaxonomic properties, together with phylogenetic distinctiveness, distinguish the four novel strains from recognized members of the family *Propionibacteriaceae*. On the basis of phenotypic, chemotaxonomic, phylogenetic and genetic data, strains R27^T, R44, R45 and R47 are classified as representatives of a new genus and novel species, *Aestuariimicrobium kwangyangense* gen. nov., sp. nov., within the family *Propionibacteriaceae*. The type strain of *Aestuariimicrobium kwangyangense* sp. nov. is R27^T (=KCTC 19182^T=JCM 14204^T).

Many novel bacterial taxa have been isolated from a variety of tidal flats on the south and west coasts of the Korean peninsula (Jung *et al.*, 2005, 2006a; Kim *et al.*, 2005; Yoon *et al.*, 2005; Lim *et al.*, 2006). In recent years, in an attempt to investigate the microbial community of oil-contaminated tidal flat sediment in Kwangyang, Korea, many bacterial strains have been isolated and characterized taxonomically (Jung *et al.*, 2006b). Four of these strains, designated R27^T, R44, R45 and R47, which contain LL-diaminopimelic acid in the cell-wall peptidoglycan, are the subject of this study. Comparative 16S rRNA gene sequence analysis indicated that the four strains are phylogenetically closely related to the family *Propionibacteriaceae*. Accordingly, the aim of the present work was to determine the exact taxonomic positions of strains R27^T, R44, R45 and R47 using a polyphasic approach that included phenotypic properties, detailed phylogenetic analysis based on 16S rRNA gene sequences, and genetic relatedness.

Oil-contaminated tidal flat sediment collected from Kwangyang, Korea, was used as the source for the isolation of bacterial strains. Sediment samples (each 0.5 mg) were inoculated in 100 ml Bushnell-Haas broth (Difco) that contained 2% (w/v) diesel oil. Cultures were incubated at 30 °C on a horizontal shaker at 150 r.p.m. Strains R27^T, R44, R45 and R47 were isolated from an enrichment culture with diesel oil-degradation activity by means of dilution plating on R2A agar (Difco). To investigate their morphological, physiological and biochemical characteristics, the four novel strains were routinely cultivated on R2A agar at 30 °C. Growth at various temperatures from 4 to 50 °C was measured on R2A agar. The pH range for growth was determined in R2A broth [prepared according to the formula of the Difco medium without agar and supplemented with 2% (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and 0.1% (v/v) trace element solution (Drews, 1983)] which was adjusted to various pH values (pH 4.5–10.5 at intervals of 0.5 pH units). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on R2A agar and on R2A agar supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Cell morphology and flagellation were examined by light microscopy (E600; Nikon) and transmission electron microscopy (TEM) by

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains R27^T, R44, R45 and R47 are DQ830982–DQ830985, respectively.

A table detailing the cellular fatty acid contents of strains R27^T, R44, R45 and R47 is available with the online version of this paper.

using cells grown on R2A agar. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Catalase and oxidase activities and hydrolysis of casein, starch, hypoxanthine, xanthine, tyrosine and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and urea and nitrate reduction were studied as described by Lanyi (1987). Acid production from carbohydrates was determined according to the method described by Leifson (1963). Utilization of various substrates for growth was assessed according to Yurkov *et al.* (1994). The API ZYM system (bioMérieux) was used to determine enzyme activity. Susceptibility to antibiotics was tested on R2A agar plates using discs containing the following concentrations of antibiotic: 10 µg ampicillin; 100 µg carbenicillin; 30 µg cephalothin; 100 µg chloramphenicol; 30 µg gentamicin; 15 µg lincomycin; 30 µg kanamycin; 30 µg neomycin; 5 µg novobiocin; 15 µg oleandomycin; 20 U penicillin G; 100 U polymyxin B; 50 µg streptomycin and 30 µg tetracycline.

Strains R27^T, R44, R45 and R47 were cultivated for 3 days in R2A broth prepared according to the formula of the Difco medium without agar and supplemented with 2% (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and 0.1% trace element solution (Drews, 1983) at 30 °C to obtain the cell biomass required for DNA extraction and for isoprenoid quinone and cell-wall analyses. Isoprenoid quinones were analysed as described previously (Komagata

& Suzuki, 1987) using reverse-phase HPLC. The isomer type of the diamino acid of the cell wall was analysed according to the method of Komagata & Suzuki (1987). For fatty acid methyl ester analysis, cell mass of strains R27^T, R44, R45 and R47 was harvested from R2A agar plates after cultivation for 3 days at 30 °C. Fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Chromosomal DNA was extracted and purified by the procedure described previously (Yoon *et al.*, 1996). The DNA G + C content was determined using the method of Tamaoka & Komagata (1984) with a modification in which DNA was hydrolysed and the resultant nucleotides were analysed by reverse-phase HPLC. Amplification of the 16S rRNA gene was performed according to the method described previously using two universal primers (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed according to Yoon *et al.* (2003). DNA-DNA reassociation was determined by the microplate hybridization method (Ezaki *et al.*, 1989) using photobiotin-labelled DNA probes.

Morphological, cultural, physiological and biochemical characteristics of strains R27^T, R44, R45 and R47 are given in the genus and species descriptions (see later) or are shown in Table 1, together with those of some phylogenetically related genera. The four novel strains were identical in all of the morphological, cultural, physiological and

Table 1. Differential characteristics of *Aestuariimicrobium kwangyangense* gen. nov., sp. nov. and phylogenetically related LL-diaminopimelic acid peptidoglycan-containing genera

Taxa: 1, *Aestuariimicrobium kwangyangense*; 2, *Propionibacterium propionicum* (data from Maszenan *et al.*, 1999; Bae *et al.*, 2006); 3, *Propioniferax* (Yokota *et al.*, 1994); 4, *Luteococcus* (Tamura *et al.*, 1994; Collins *et al.*, 2000, 2003); 5, *Tessaracoccus* (Maszenan *et al.*, 1999). All species are Gram-positive and non-motile. +, Positive; -, negative; v, variable reaction; ND, not determined.

Characteristic	1	2	3	4	5
Origin	Tidal flat	Human oral cavity and cervicovaginal secretion	Human epidermis	Soil or human clinical specimen	Activated sludge
O ₂ metabolism	Aerobic	Facultatively anaerobic	Facultatively anaerobic	Facultatively anaerobic	Facultatively anaerobic
Cell morphology	Short rods or cocci	Pleomorphic rods	Pleomorphic rods	Cocci or pleomorphic rods	Cocci
Growth temperature range (°C)	4–40	30–37	10–40	ND	20–37
Optimal temperature for growth (°C)	30	35–37	37	26–28*	25
Optimal pH range for growth	7.5–8.5	ND	7.0	ND	7.5
Catalase	+	–	+	+	+
Oxidase	–	ND	+	+	–
Nitrate reduction	+	+	+	v	+
Major menaquinone(s)	MK-9(H ₄)	MK-9(H ₄)	MK-9(H ₄)	MK-9(H ₄)	MK-9(H ₄), MK-7(H ₄)
Major fatty acid(s)	anteiso-C _{15:0}	anteiso-C _{15:0} , iso-C _{15:0}	anteiso-C _{15:0} , iso-C _{15:0}	C _{17:1} , C _{15:1} , C _{16:1} (C _{16:1} , C _{17:1} , C _{18:1})*	anteiso-C _{15:0}
DNA G + C content (mol%)	68.8–69.2	63–65	59–63	64–67	74

*Data for *Luteococcus japonicus* are from Tamura *et al.* (1994).

biochemical characteristics tested in this study, except with regard to the utilization of trehalose, benzoate and pyruvate and in the activities of naphthol-AS-BI-phosphohydrolase and β -glucuronidase. The almost complete 16S rRNA gene sequences of the four strains determined in this study were identical and comprised 1472 nucleotides. Phylogenetic analysis based on 16S rRNA gene sequences showed that strains R27^T, R44, R45 and R47 formed an independent lineage of descent within the family *Propionibacteriaceae* of the suborder *Propionibacterineae* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, the four novel strains joined the clade comprising the genera *Propionibacterium*, *Tessaracoccus*, *Brooklawnia*, *Propionimicrobium* and *Luteococcus* (Fig. 1). Strains R27^T, R44, R45 and R47 exhibited 16S rRNA gene sequence similarity values of less than 94.5% with respect to other species used in the phylogenetic analysis (Fig. 1). The 16S rRNA gene sequence similarity values between the four novel strains and phylogenetically related genera were as follows: *Propionibacterium* (93.3–90.0%), *Tessaracoccus* (93.3%), *Brooklawnia* (94.1%), *Propionimicrobium* (92.0%), *Luteococcus* (94.0–94.5%) and *Propioniferax* (92.4%). Strains R27^T, R44, R45 and R47 exhibited a mean level of DNA–DNA relatedness of 91.2–97.3% when their DNA was used individually as labelled DNA probes, indicating that the four novel strains are members of the same genomic species (Wayne *et al.*, 1987). In view of the combined phenotypic, phylogenetic and genetic similarities, strains R27^T, R44, R45 and R47 are considered to represent members of the same species.

Strains R27^T, R44, R45 and R47 contained LL-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan which is characteristic of wall chemotype I *sensu* Lechevalier & Lechevalier (1970). The four strains contained a tetrahydrogenated menaquinone with nine isoprene units [MK-9(H₄)] as the predominant isoprenoid quinone. The predominant menaquinone type [MK-9(H₄)] of the four novel strains was the same as that of members of the family *Propionibacteriaceae* (Tamura *et al.*, 1994; Yokota *et al.*, 1994; Nakamura *et al.*, 1995; Stackebrandt

et al., 2002; Collins *et al.*, 2003; Bae *et al.*, 2006). The cellular fatty acid profiles of strains R27^T, R44, R45 and R47 are shown in Supplementary Table S1 (available with the online version of this paper). The major component was anteiso-C_{15:0} (46.4–67.9%). Strain R47 was slightly different from the three other strains in the proportions of anteiso-C_{15:0} and C_{16:0} (see Supplementary Table S1). The DNA G+C contents of strains R27^T, R44, R45 and R47 were 68.8, 69.1, 69.0 and 69.2 mol%, respectively.

Strains R27^T, R44, R45 and R47 could be clearly distinguished from other members of the family *Propionibacteriaceae* by differences in chemotaxonomic properties (Pitcher & Collins, 1991; Yokota *et al.*, 1994; Maszenan *et al.*, 1999; Stackebrandt *et al.*, 2002; Collins *et al.*, 2003; Bae *et al.*, 2006). The genus *Tessaracoccus* contains MK-9(H₄) and MK-7(H₄) as the predominant menaquinones (Maszenan *et al.*, 1999). Strains R27^T, R44, R45 and R47 differ from the genera *Propionimicrobium* and *Brooklawnia* and *Propionibacterium freudenreichii* which contain lysine or *meso*-diaminopimelic acid as the diamino acid in position 3 of the peptidoglycan (Table 1). The fatty acid profiles, particularly major fatty acids, distinguish the four novel strains from some phylogenetically related genera, although there may be differences in the proportions of some fatty acids, probably due to differences in cultivation conditions and extraction procedures (Table 1). In particular, strains R27^T, R44, R45 and R47 are clearly distinguished from the genus *Luteococcus* by differences in major fatty acids (Table 1). The novel four strains contain anteiso-C_{15:0} as the major fatty acid, whereas members of the genus *Luteococcus* synthesize predominantly monounsaturated fatty acids, including C_{17:1}, C_{16:1}, C_{15:1} or C_{18:1} (Tamura *et al.*, 1994; Collins *et al.*, 2000, 2003; Supplementary Table S1). Low 16S rRNA gene sequence similarity values between the four novel strains and other members of the family *Propionibacteriaceae*, together with differential phenotypic properties, suggest that strains R27^T, R44, R45 and R47 constitute a novel genus and species within the family *Propionibacteriaceae*, for which the name *Aestuariimicrobium kwangyangense* gen. nov., sp. nov. is proposed.

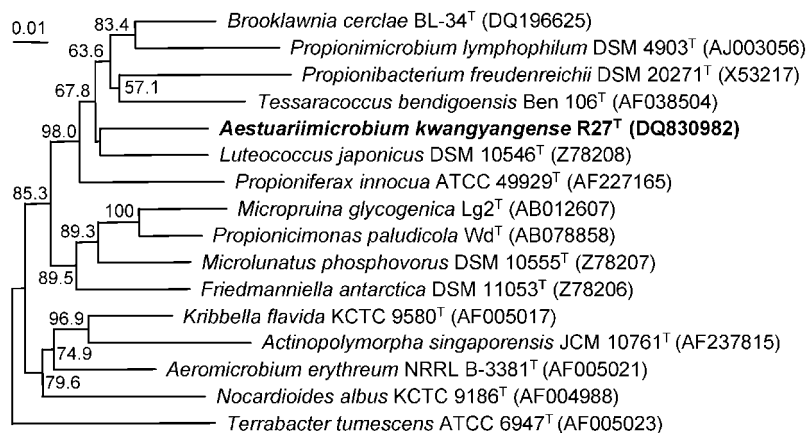


Fig. 1. Neighbour-joining tree showing the phylogenetic positions of *Aestuariimicrobium kwangyangense* gen. nov., sp. nov. R27^T and other related taxa based on 16S rRNA gene sequences. Only bootstrap values (expressed as percentages of 1000 replications) of greater than 50% are shown at the branching points. *Terrabacter tumescens* ATCC 6947^T (GenBank accession number, AF005023) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Description of *Aestuariimicrobium* gen. nov.

Aestuariimicrobium (Aes.tu.a.ri.i.mi.cro'bi.u.m. L. n. *aestuarium* part of the sea coast which, during the flood-tide, is overflowed, but at ebb-tide is left covered with mud or slime, a tidal flat; N.L. n. *microbium* microbe; N.L. neut. n. *Aestuariimicrobium* a microbe isolated from tidal flat).

Cells are Gram-positive, non-flagellated, short rods or cocci (0.6–1.2 × 1.2–2.0 μm). The predominant menaquinone is MK-9(H₄). The cell-wall peptidoglycan contains LL-diaminopimelic acid. The major cellular fatty acid (>10% total fatty acids) is anteiso-C_{15:0}. The DNA G+C content is 68.8–69.2 mol%. Phylogenetically, the genus *Aestuariimicrobium* is a member of the family *Propionibacteriaceae* within the suborder *Propionibacterineae* of the order *Actinomycetales*. The type species is *Aestuariimicrobium kwangyangense*.

Description of *Aestuariimicrobium kwangyangense* sp. nov.

Aestuariimicrobium kwangyangense (kwang.yang.en'se. N.L. neut. adj. *kwangyangense* pertaining to Kwangyang, Korea, from where the type strain was isolated).

Exhibits the following properties in addition to those given in the genus description. Colonies on R2A agar are circular, convex, smooth, yellow and 0.8–1.0 mm in diameter after 3 days incubation at 30 °C. Growth does not occur under anaerobic conditions on R2A agar or on R2A agar supplemented with nitrate. Catalase-positive, oxidase-negative and urease-negative. Growth occurs at 4 and 40 °C with an optimum temperature of 30 °C. Optimal pH for growth is 7.5–8.5. Aesculin, casein and Tweens 20, 40, 60 and 80 are hydrolysed, but starch, hypoxanthine, xanthine and tyrosine are not. Nitrate is reduced to nitrite. D-Glucose, D-fructose, D-galactose, D-cellobiose, D-mannose, D-xylose, sucrose, maltose and salicin are utilized as sole carbon and energy sources, but L-arabinose, acetate, citrate, succinate, L-malate, formate and L-glutamate are not utilized. Utilization of trehalose (only strain R47 is negative) and benzoate and pyruvate (only strain R44 is positive) are variable. Acid is produced from D-sorbitol, D-xylose, D-fructose, melibiose, L-arabinose, D-melezitose, D-glucose, D-galactose, L-rhamnose, D-mannose, D-cellobiose, lactose, sucrose, maltose, trehalose and D-raffinose, but not from *myo*-inositol or D-mannitol. Susceptible to penicillin G, chloramphenicol, ampicillin, cephalothin, novobiocin, tetracycline and carbenicillin, but not to polymyxin B, streptomycin, gentamicin, kanamycin, lincomycin, oleandomycin or neomycin. Using the API ZYM system (bioMérieux), esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase are present, but alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Naphthol-AS-BI-phosphohydrolase and

β-glucuronidase activities (only type strain is negative) are variable.

The type strain, R27^T (=KCTC 19182^T=JCM 14204^T), was isolated from oil-contaminated tidal flat sediment of Kwangyang, Korea.

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